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Increased sensitivity of HIV variants selected by attachment inhibitors to broadly neutralizing antibodies

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ABSTRACT

Treatment with HIV attachment inhibitors (AIs) can select for escape mutants throughout the viral envelope. We report on three such mutations: F423Y (gp120 CD4 binding pocket) and I595F and K655E (gp41 ectodomain). Each displayed decreased sensitivity to the AI BMS-488043 and earlier generation AIs, along with increased sensitivity to the broadly neutralizing antibodies 2F5 and 4E10, without affecting the rate of viral entry or sensitivity to the entry inhibitors AMD-3100 and Enfuvirtide. We also observed that I595F did not substantially increase envelope sensitivity to HIV-infected patient sera. Based on these observations, we propose that although F423Y, I595F and K655E may all affect the presentation of the 2F5 and 4E10 epitopes, natural immune mimicry is rare only for the I595F effect. Thus, it seems that in addition to restricting AI resistance development, incorporation of I595F into an appropriate vehicle could elicit a novel antiviral response to improve vaccine efficacy.

Introduction

The HIV envelope is located on the virion surface and is constantly subjected to host immune selection pressure, as it is the target of the majority of circulating HIV neutralizing antibodies present in infected patient sera (Li et al., 2009). Natural selection drives HIV to constantly evolve to evade the host immune response (Parren et al., 1999; Watkins et al., 1996), aided by the flexible and heterogeneous nature of the envelope, a thick carbohydrate shield, and hidden, discontinuous epitopes (Kwong et al., 1998). All of these characteristics not only present a high hurdle in the maintenance of an effective host immune response, but also a challenge in designing an effective, broad-spectrum vaccine. Although patient sera have been shown to neutralize autologous and/or heterologous virus (Deeks et al., 2006; Parren et al., 1999), only a handful of broadly reactive neutralizing antibodies have been identified (Burton et al., 1994; Haynes and Montefiori, 2006). Strategies to induce the exposure of cross-reactive neutralizing antibody epitopes would therefore greatly enhance the chances for the success of a humoral vaccine design (Montero et al., 2008; Zhang and Dimitrov, 2007; Zhang et al., 2004b,c).

HIV attachment inhibitors (AIs) belong to a novel class of oral, small-molecule HIV antiretrovirals (Guo et al., 2003; Ho et al., 2005; Lin et al., 2003). These inhibitors bind directly to the HIV envelope gp120 protein, block CD4 receptor binding, induce conformational changes at CD4 and CCR5 binding regions, and interfere with downstream entry events (Ho et al., 2005, 2006). Similar to other classes of HIV inhibitors, drug-resistant viruses emerge after exposure to AIs (Colonno et al., 2004; Gong et al., 2000; Larder et al., 1995; Lin et al., 2003; Madani et al., 2004). The complexity of Env structure/function and the allosteric nature of some forms of AI escape lead to the postulate that AI resistance could affect susceptibility to neutralizing antibodies or other classes of entry inhibitors (Ho et al., 2006; Kwong et al., 1998; Sanders et al., 2008; Wyatt et al., 1998). This hypothesis was tested using the broadly neutralizing antibodies 4E10 and 2F5 (Binley et al., 2006; Calarese et al., 2005; Nakowitsch et al., 2005; Zhang et al., 2004c; Zhou et al., 2007; Zwick et al., 2001, 2005), as well as the fusion inhibitor Enfuvirtide (Manfredi and Sabbatani, 2006; Wild et al., 1992) and the CXCR4 coreceptor inhibitor, AMD-3100 (Khan et al., 2007; Schols et al., 1997). In this report, the effect of AI-resistant mutations on the sensitivity of virus to sera from HIV-

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infected patients was examined, and the potential utility and impact of these changes on vaccine design and therapy are discussed.

Results

Selection of F423Y, I595F, and K655E by attachment inhibitors

Attachment Inhibitors (AI) have evolved from early leads such as BMS-313216 (Fig. 1A) to the more potent prototype BMS-378806 (Fig. 1B). However, BMS-378806 failed to achieve target exposures in humans (unpublished observations), so BMS-488043 (Fig. 1C), with improved *in vitro* antiviral activity, a longer half-life and higher plasma concentrations in humans was developed (Alexander et al., 2009; Wang et al., 2009). To understand the effect of AI-resistant mutations on the immune response to envelope, BMS-313216 and BMS-378806 were used to select virus with decreased sensitivity to these compounds. Treatment of HIV-LAI with 60 nM (~2× EC_{50}) of BMS-313216 produced a population that displayed >1000-fold resistance (Table 1). Twenty-three independent clones were acquired from this pool, and sequence analyses revealed that 16/23 and 15/23 clones contained I595F and/or F423Y, respectively, and that 7/23 clones contained both mutations (Table 1). Analogous experiments with BMS-378806 selected I595F in the absence of F423Y (6/8 clones; Table 1), as well as K655E (6/17 clones; Table 1).

Effect of F423Y, I595F, and K655E on HIV entry inhibitor sensitivity

We suspected that mutations selected by one AI would confer resistance to another compound of this class. To test this premise F423Y, I595F, and K655E were introduced into the LAI background in either an envelope expression vector or a full-length infectious HIV clone. The former was used to coat pseudoviruses that were used in a single-cycle format in which HeLa CD4 cells served as the target. The latter was used to generate HIV recombinants to infect MT-2 cells in a multiple-cycle assay format. These studies showed that these substitutions generated by treatment with earlier generation AIs also conferred resistance to BMS-488043 in both the single- (Fig. 2A and Table 2) and multiple-cycle (Table 3) assay formats.

Two of the mutations (I595F, and K655E) in our study reside in the ectodomain of gp41. We reasoned that these changes could affect envelope sensitivity to the broadly neutralizing antibodies (bNAbs) 2F5 and 4E10, the epitopes of which are also located in this region of the envelope (Montero et al., 2008; Zwick et al., 2001). Thus, pseudotyped viruses with these substitutions were examined for their ability to be neutralized by 2F5 and 4E10. The single-cycle assay revealed that not only did the gp41 mutations show enhanced neutralization sensitivity to these antibodies, but surprisingly, so did the F423Y substitution (>30-fold effect), although it is located in the CD4 binding pocket of gp120 (Figs. 2B and C, and Table 2). Comparable results were obtained using the fully infectious multi-cycle assay (Table 3).

We expanded our studies to address the question of whether the altered sensitivities of F423Y, I595F, and K655E for BMS-488043 and the bNAbs applied to additional HIV entry inhibitors. Experiments with inhibitors targeting the CXCR4 coreceptor (AMD-3100) and membrane fusion (Enfuvirtide) showed that pseudoviruses containing the gp41 mutants maintained sensitivity that was similar to that of wild-type LAI (Table 4). The F423Y mutant displayed an increase in sensitivity to these inhibitors but the effect was only 2.5-fold (Table 4), which was not comparable with the 8- to 100-fold effect observed for increased 2F5 and 4E10 sensitivity (Table 2).

Neutralizing sensitivity of F423Y, I595F, and K655E to HIV-infected patient sera

We next examined whether F423Y, I595F, or K655E envelopes would have altered sensitivity to sera from HIV infected individuals. F423Y, I595F and K655E are either rare (2/157 for K655E, 3/157 for I595F) or absent (F423Y) among clade B isolates (http://www.hiv.lanl.gov/content/index); one might expect no effect of these mutations against AI-naive serum. To test this premise, wild-type LAI or mutants HIV carrying the F423Y, I595F, or K655E substitutions were evaluated against 24 heat-treated serum samples from such individuals. Surprisingly, these studies revealed that the K655E recombinant displayed a >5-fold increase in sensitivity for 87.5% (21/24) of the samples evaluated, with 42% (10/24) of the samples displaying a >20-fold increase in sensitivity (Table 5). For F423Y, a >5-fold elevation in neutralization sensitivity was observed for 50% (12/24) of the samples tested, with a >20-fold increase observed for 17% (4/24) of the samples tested (Table 5). Only the I595F recombinant had a minimal effect on sera neutralization characteristics, displaying an increase in sensitivity of >5-fold for just 8.3% (2/24) of the samples evaluated (Table 5).

Cell–cell fusion mediated by the HIV envelope

To assess if these mutations could affect the rate of envelope mediated cell entry we employed an assay that quantified Enfuvirtide-sensitive fusion events between calcein AM- (green) labeled HeLa cells expressing wild-type or mutant envelope and CMTMR-

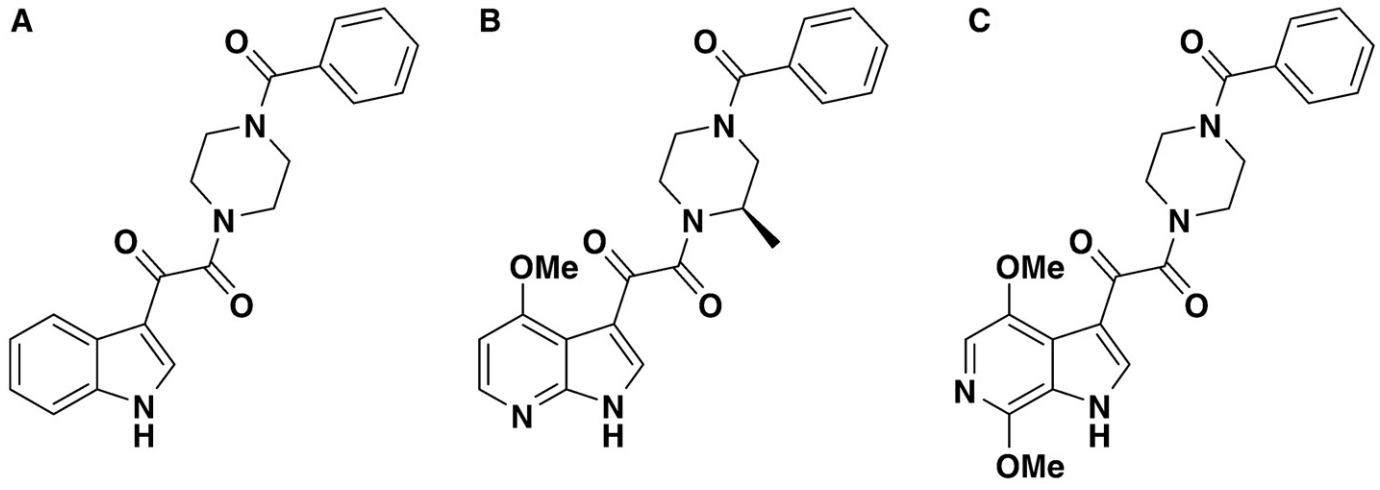

Fig. 1. (A) BMS-313216, (B) BMS-378806, and (C) BMS-488043.

Table 1
Sequence of clones selected by attachment inhibitor treatment.

AI	Fold resistance	Envelope Mutation	Frequency
BMS-313216	**>1396**	E267G/**F423Y**/R444K/I596F	**1/23**
		A266V/**F423Y**/**I595F**/E620E	**1/23**
		W96R/**F423Y**/E560G/**I595F**	**1/23**
		D57G/D78N/T303I/S411N/**F423Y**/F685L	**1/23**
		N92D/S306G/R315G/**I595F**/L800P/K808R	**1/23**
		N339S/**F423Y**/Q509R/**I595F**/N816S	**1/23**
		T31A/**F423Y**/**I595F**/G738E	**1/23**
		S264N/**F423Y**/**I595F**	**1/23**
		V68A/T303I/S398N/A541T/I595S	**1/23**
		F93L/Q203R/N301K/**F423Y**/**I595F**	**1/23**
		F423Y/**I595F**	**1/23**
		F175L/S306G/S387P/S398G/**I595F**	**1/23**
		F423Y/S546P/R740G/R848S	**1/23**
		E32K/V68A/T303I/Q352R/A541T/**I595F**	**1/23**
		V84I/**F423Y**/A541T	**1/23**
		L21P/N80D/E171K/A224V/F317L/ **F423Y**/**I595F**	**1/23**
		V68A/**I595F**	**1/23**
		E153G/M301K/L342V/**I595F**	**1/23**
		S306G/**F423Y**/S546P	**1/23**
		E106K/L125F/S546P	**1/23**
		T303I/S334N/**F423Y**/A541T/D741G	**1/23**
		A137T/S306G/G324R/**I595F**	**1/23**
		F423Y/P470S/**I595F**	**1/23**
BMS-378806	**1941**	T303A/M434I/**I595F**/E648G	**1/8**
		Y61H/N301K/L349I/G441R/**I595F**/S813G	**1/8**
		I595F	**1/8**
		M434I/C837Y/A839V	**1/8**
		M434I/**I595F**/V698A/R846G	**1/8**
		L86M/M434I/C445Y/Q507R/**I595F**	**1/8**
		G152E/I165M/**I595F**/K655R	**1/8**
		M434I/R469G/R542G/**I595F**	**1/8**
BMS-378806	**169**	L116P/S440R/**K655E**	**1/17**
		V68A/S164G/T140I/G366E/M426L	**1/17**
		V68A/G152E/M426L/Q562R	**1/17**
		M426L/A607T	**1/17**
		V68A/S195R/M426L	**1/17**
		G152E/S334R/F376L/F383S/M434I/ S767N/I777V/N816S	**1/17**
		E351K/M426L/S440R/S767G	**1/17**
		L21P/V68A/M426L/T569A/H842R	**1/17**
		V68A/M426L/S440R/T605I/**K655E**/ Δ822-823	**1/17**
		E351K/M426L/M434I/L523S/**K655E**/I830V	**1/17**
		V68A/S142N/M426L/**K655E**	**1/17**
		E351K/M426L/M434I/A612T/I684M/ R696G/L765R	**1/17**
		V68A/E351K/M426L/M434V/V506M	**1/17**
		V68A/F175L/M426L	**1/17**
		V68A/D107N/S144N/M434V/**K655E**	**1/17**
		V68A/M434V/**K655E**	**1/17**
		V68A/E351K/M426L	**1/17**

Table 2
HIV pseudovirus sensitivity to BMS-488043 and bNAbs. The values summarize the data in Fig. 2. Values differing from WT by >5-fold are in bold.

Virus	BMS-488043			2F5			4E10		
	EC_{50} (nM)	STD	Fold WT	EC_{50} (μg/ml)	STD	Fold WT	EC_{50} (μg/ml)	STD	Fold WT
WT	0.98	0.20		1.88	0.32		9.29	1.44	
F423Y	**10.9**	1.65	**11**	**0.06**	0.01	**0.03**	**0.72**	0.09	**0.08**
I595F	**19.0**	4.47	**19**	**0.22**	0.03	**0.12**	**0.27**	0.06	**0.03**
K655E	**6.06**	0.96	**6**	**0.02**	0.01	**0.01**	**0.22**	0.05	**0.02**

(red) labeled HeLa cells expressing CD4 and HIV coreceptors. Fusion events (which served as a model for HIV entry) were identified by altered fluorescence (orange, Fig. 3), allowing their rate of appearance to be quantified. These analyses revealed that in comparison to cultures expressing wild-type LAI treated with Enfuvirtide (background nonspecific fusion), wild-type LAI and the mutant envelopes induced a similar multiple over the frequency of nonspecific events (Table 6). Moreover, the levels of fusion prevalence were also comparable for these different envelopes (Table 6). From these data, we conclude that the mutations do not substantially affect the rate of HIV entry into cells and that such a mechanism could not explain the observed level of sensitivity to the bNAbs.

Discussion

The envelope coats HIV particles and consequently, is the primary target of humoral immunity. HIV vaccine strategies aimed at eliciting antibodies that can neutralize a broad range of HIV sequences have been unsuccessful. Thus, novel approaches that could increase the prospects of eliciting effective immunity are indicated. We sought to test if mutations selected by AIs could alter the conformation of the envelope to increase the sensitivity to bNAbs, and potentially, the immunogenicity of vaccine vectors that incorporated these mutations. In characterizing such mutations, it was surprising that these compounds selected for I595F and K655E, which are located in the ectodomain of gp41 (Table 1), since AIs dock in the CD4 binding pocket (Ho et al., 2006). Equally surprising was our observation that like 1595F and K655E, the F423Y mutant, residing in an area of gp120 proximal to the CD4 binding cavity (Chen et al., 2005a,b), increased sensitivity to the bNAbs 2F5 and 4E10 (Fig. 2; Tables 2 and 3). These findings highlight the complex nature of envelope structure/function and suggest that the development of AI resistance in treated patients could be restricted by specific host immune responses. This is consistent with recent reports in which sequence changes in the envelope that confer resistance to other HIV entry inhibitors can increase the accessibility of some neutralizing antibody epitopes (Hu et al., 2007; Pugach et al., 2008; Reeves et al., 2005; Vermeire et al.,

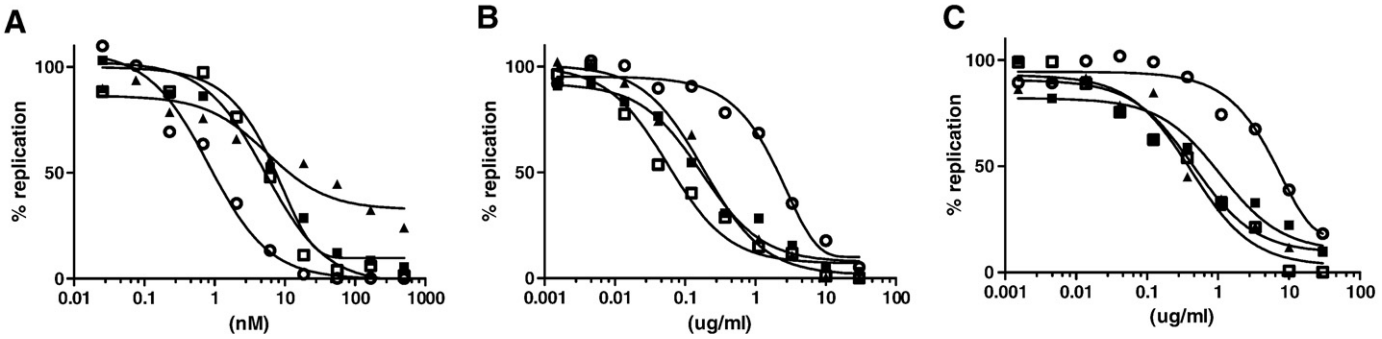

Fig. 2. Inhibition of HIV pseudoviruses entry. Pseudovirions coated with wild-type (open circles) or mutant [F423Y (filled squares), I595F (filled triangles), and K655E (open squares)] envelopes treated with (A) BMS-488043, (B) 2F5, and (C) 4E10. Error bars have been omitted for clarity (the data are summarized in Table 2). Curve fitting was performed with nonlinear regression analysis using Prism software.

Table 3
Whole HIV sensitivity to BMS-488043 and bNAbs. Values differing from WT by >5-fold are in bold.

Virus	BMS-488043			2F5			4E10		
	EC_{50} (nM)	STD	Fold WT	EC_{50} (μg/ml)	STD	Fold WT	EC_{50} (μg/ml)	STD	Fold WT
WT	0.13	0.07		2.96	1.38		1.78	0.50	
F423Y	**1.15**	0.65	**8**	**0.20**	0.03	**0.1**	**0.20**	0.09	**0.1**
I595F	**0.74**	0.28	**6**	**0.47**	0.11	**0.2**	**0.06**	0.02	**0.03**
K655E	**0.65**	0.04	**5**	**0.47**	0.14	**0.2**	**0.07**	0.00	**0.04**

Table 5
Neutralizing activity of HIV-infected patient serum.

Virus	Fold increase in neutralizing sensitivity	Frequency
F423Y	>20	4/24
	5–20	8/24
I595F	>20	0/24
	5–20	2/24
K655E	>20	10/24
	5-20	11/24

2009), thus illustrating a potentially unique benefit of targeting this step in the HIV life cycle.

The increased sensitivity to 2F5 and 4E10 conferred by F423Y, I595F, and K655E seems to be specific in that they did not increase sensitivity to the other entry inhibitors tested (Table 4). However, these mutations do not affect other properties of gp160, as F423Y, I595F, and K655E do not seem to affect the rate of HIV cell entry (Table 6), which we have modeled with a cell–cell fusion assay (Fig. 3). Consequently, it does not seem that increased sensitivity to bNAbs is due to extended exposure of the envelope to the bNAbs. Instead, we favor a model in which these mutations have an effect similar to mutations in the 2F5 and 4E10 epitopes, which also increase sensitivity to their respective bNAb without affecting sensitivity to Enfuvirtide (Zwick et al., 2005). It is thought that they operate by altering the presentation of these epitopes through conformational changes in this domain. We suspect that the mutants in our study similarly affect 2F5 and 4E10 epitope presentation/conformation, albeit through an allosteric mechanism. This model is consistent with previous observations in which the presentation of the 2F5 and 4E10 epitopes is context-dependent; underscored by the limited success that has been achieved in eliciting nAbs using 2F5 epitope mimics (Zhang et al., 2004a).

Reinforcing the relevance of context in epitope presentation are data showing that the sensitivity of HIV to neutralization can be influenced by the properties of virus producer cells as well as the type of recipient/reporter system employed to determine viral production/entry inhibition. In particular, the use of reporter cell lines can lead to *in vitro* results that do not correlate with *in vivo* data. To ensure that our data were representative, we employed two distinct systems. In one, pseudovirus was produced from 293T cells, and entry inhibition was tested in HeLa CD4 cells in which firefly luciferase expression was driven by the HIV LTR. In the other, infectious HIV produced from the T cell line MT-2 was used to infect this same line. The data generated from these two formats were comparable for BMS-488043, 2F5, and 4E10 sensitivities (Tables 2 and 3), suggesting that the observed activities are representative of effects *in vivo*. In contrast, our mutants behaved differently against the bNAb 2G12 in the two formats (data not shown), and therefore, these data were not included in this report. Interestingly, it was the 2G12 molecule in which issues with reporter cell lines were previously documented (Mann et al., 2009).

Although F423Y, I595F, and K655E exhibit similar characteristics with respect to entry inhibitor sensitivity, they had distinct profiles with regards to sensitivity to AI naive HIV infected patient sera (Table 4). In contrast to F423Y and K655E, I595F displayed sensitivity that was comparable to wild type LAI; only showing increased sensitivity to 2/24 samples (Table 4), with no substantial decrease in sensitivity for any of the patient sera (data not shown). We interpret these data to mean that these patients, who have developed immunity to their circulating HIV pool, have not developed immunity that mimics the effect of this mutation. This was the anticipated outcome for all these mutations since they are rarely seen in Clade B isolates and since these sera are from patients that are AI naive. We cannot provide an explanation for the unexpected behavior of F423Y and K655E, but it does not seem that they would enhance therapeutic immunity. On the other hand, our observations imply that inclusion of I595F in a vaccine vehicle could induce a novel and possibly broadly neutralizing immune response to HIV. Such a response might improve the efficacy of a prophylactic vaccine or might be utilized to augment natural immunity as a therapeutic option.

Our results show that applying AI selection pressure to the viral entry process can lead to mutations that increase envelope sensitivity to broadly neutralizing epitopes. These specific AI selected conformations could potentially provide novel immunogenic epitopes for HIV vaccine approaches. Moreover, the AI selected envelopes presented here could serve as a tool to gain a better understanding of the HIV entry process and relevant therapeutic applications.

Methods

Reagents

2F5 and 4E10 were obtained from POLYMUN Scientific Immunbiologische Forschung GmbH (Austria, Vienna). Sera from HIV-infected patients were purchased from Bioreclamation Inc. (Hicksville, NY). All samples were from patients ≥2 months off antiretroviral

Table 4
HIV pseudovirus sensitivity to HIV entry inhibitors. Values expressed as fold change in comparison to wild type.

Virus	AMD-3100	Enfuvirtide
F423Y	0.4	0.4
I595F	1.0	1.0
K655E	1.0	1.1

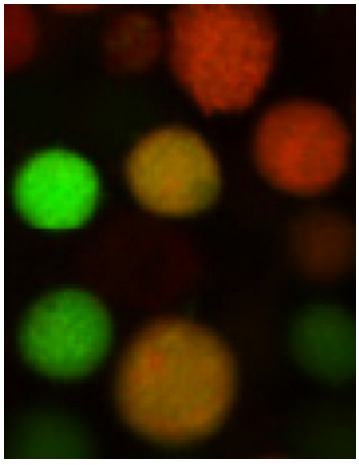

Fig. 3. HIV envelope mediated cell–cell fusion. Calcein AM-labeled HeLa cells expressing the HIV envelope (green, left), CMTMR-labeled HeLa cells expressing CD4 and HIV coreceptors (red, upper right), and fused cells (orange, middle).

Table 6
Envelope-mediated cell–cell fusion.

Virus	Fold over Enfuvirtide-treated LAI	Fusion % (20 min coculture)
Wild-type	8.37	27.1
F423Y	8.27	32.6
I595F	6.35	23.6
K655E	6.95	27.3

treatment. HIV entry inhibitors were prepared at Bristol Myers Squibb (Wallingford, CT).

Attachment inhibitor resistance selection

To identify envelope changes that decrease sensitivity to an AI, HIV-LAI was initially treated with 2× the EC_{50} of compound. Infected cells were cultured until cytopathic effects were observed. At that time, the EC_{50} of the resulting strain was determined as previously described (Lin et al., 2003). If high-level resistance (>100-fold over wild type) was observed, RT–PCR of the isolated viral RNA was performed, and the sequence of individual clones was determined. If high-level resistance was not attained, the concentration of AI was increased 2- to 10-fold, and the process was repeated until high-level resistance was observed. Mutations that appeared in >1/3 of the clones were considered signatures of resistance.

Preparation of pseudotyped viruses and recombinant HIV

Functional HIV envelope expression plasmids were constructed by PCR with LAI *env* coding regions inserted into either pCDNA or pTRE vectors. Mutations within the *envelope* gene were introduced by site-directed mutagenesis using the QuikChange Site-directed Mutagenesis Kit II (Stratagene, La Jolla, CA). To produce a specific HIV envelope pseudotyped virus, the desired HIV envelope expression plasmid and an HIV core expression plasmid [pLAI Luc (Δenv)] (Lin et al., 2003) were cotransfected into 293T cells using the Lipofectamine reagent (Invitrogen, Carlsbad, CA). Transfected 293T cells were incubated for 48 h, and virus was harvested. Alternatively, these mutations were introduced into the full-length infectious HIV clone pLAI.2 (NIH AIDS Reagent Repository, Rockville, MD) using the QuikChange Site-directed Mutagenesis Kit II. This DNA was also transfected into 293T cells from which infectious recombinant HIV was harvested.

HIV neutralization assay

Susceptibility to neutralization was evaluated using an envelope pseudotyped virus infection assay. HeLa CD4 cells were seeded (10^4/well) 1 day before assay in selection-free medium [Dulbecco modified Eagle medium (DMEM) and 10% fetal bovine serum (FBS)]. LAI (wild-type or mutant) pseudotyped virus was added to cells in the presence of inhibitor or patient serum. After a 3-day incubation, cell-associated luciferase activity was determined with a Luciferase Reporter Gene Assay (Roche, Basel, Switzerland). Alternatively, an infectious HIV assay was utilized. Virus diluted in RPMI (10% FBS) was preincubated with 3-fold serially diluted inhibitor. After 1 hour, MT-2 cells were added at 1:1 (vol./vol.) ratio to the final density of 0.1 M cells/ml and an MOI of 0.005. On day 5 after infection, the virus yield (p24, Roche) was determined in the culture supernatant.

Cell–cell fusion assay

To assess the rate of envelope mediated entry for wild-type LAI and recombinant envelopes containing the F423Y, I595F, and K655E mutations, a cell–cell fusion assay was utilized (Chien et al., 2009; Rawat et al., 2004). HeLa cells were cotransfected with pTet-Off plus and either pTRE-LAI wt, F423Y, I595F, or K655E using Lipofectamine 2000 (Invitrogen). At 24 to 48 hours after transfection, these cells were stained with the fluorescent dye Calcein AM following the manufacturer's protocol (Invitrogen). HeLa cells that express CD4 and HIV coreceptors were stained with CMTMR (Invitrogen). To promote fusion between these two cell populations, cells were mixed together at a 1:1 ratio and incubated at 37 °C to determine the rate of fusion. Fusion events were detected using an Evotech Opera confocal imaging system (PerkinElmer, Campbell, CA). Confocal images were analyzed, and fusion events were quantified (based on a minimum of 300 events) using a Cell–Cell Fusion Script algorithm following the manufacturer's recommendations (PerkinElmer).

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